

Effect of Delay in Anterior or Posterior Amputation on Regeneration of Short Fragments of Planaria

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ABSTRACT—Regeneration of fragments obtained by two transverse amputations, with the second amputation being delayed for various periods of time after the first, was examined in the freshwater planarian *Dugesia japonica*. When the two amputations were performed simultaneously, all of the fragments isolated from pre- and postpharyngeal regions regenerated as normal worms. However, when the second amputation was performed later than the first, a delay from 12 hours to 3 days occasionally caused the reversal of axial polarity and the regeneration of bipolar heteromorphs. In the first group, which consisted of fragments in which the anterior amputation was performed later than the posterior one, bipolar tails were obtained predominantly and the incidence of such regenerates increased considerably with a delay of 1 to 2 days, with the exception of cases in which bipolar heads were formed on the fragments isolated from the prepharyngeal region. In the second group, which consisted of fragments in which the posterior amputation was performed later than the anterior one, bipolar heads were obtained, and their incidence also increased considerably with a delay of 1 to 2 days between amputations. However, when the second amputation was delayed for more than four days, no bipolar heteromorphs were obtained in either group. Based on these results, a discussion is presented on the role of the anterior and posterior cut ends of a regenerating fragment in body patterning.

INTRODUCTION

Every section of the body of a freshwater planarian can normally regenerate a head from the anterior cut end and a tail from the posterior cut end and, thus, it develops into a new individual with normal proportion. The mechanism that allows the sections to maintain the information related to the original axial polarity has been investigated and discussed for a long time. According to up-to-date model of biological pattern formation of planaria [1], it is suggested that the planarian body and regenerating sections are a system with an organizing center at each end, as in the case of the body of hydra [2]. In other words, the tail and regenerating tail are a second organizing area, in addition to the head and regenerating head. Chandebois [3] showed that a tail part was not remolded by a head piece in contact with it but,

rather, the tail part brought about morphallaxis of the head piece. Our previous transplantation experiments [4, 5, 6] also indicated that a tail has a considerable capacity for determining polarity, as a head does. These experimental results provide strong support for Meinhardt's suggestion [1]. On the other hand, it was ascertained by several groups of researchers that determination of whether cut surfaces after amputation will regenerate a head or a tail occurs soon after amputation, when a blastema has not yet formed [7-13]. The ingenious experiment of Sengel [14] showed that the early blastema, separated from the stump and cultured *in vitro*, can differentiate into a head or a tail according to the original position of the fragments. These findings suggest that new positional information within the body section has been already established prior to the formation of the blastema and, possibly, that the stump tissue plays an important role to the determination of the blastema. So, such dynamic actions should be occurred not only at the anterior cut end but also at the posterior

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one.

Very short fragments of the planarian body tend to become bipolar heteromorphs, namely, bipolar heads, referred to as Janus heads, or bipolar tails, Janus tails, as reported in *Dugesia lugubris* and *Dugesia maculata* by Morgan [15, 16], in *Dugesia tigrina (maculata)* by Child [17] and in *Polycelis sapporo* by Watanabe [18]. However, if the second amputation that engenders the fragments is delayed for some periods of time after the first amputation, even short fragments that often develop into bipolar-head regenerates never produce such heteromorphs [18]. If the tail is an organizing area, as mentioned above, and if it plays some role in determining the blastema, does a similar event occur in the case of the bipolar tail? To examine this question, we monitored the regeneration of short fragments, making anterior and posterior transverse amputations at different times.

MATERIALS AND METHODS

Specimens of the freshwater planarian *Dugesia japonica* were collected from a stream in Kanazawa City. Asexual worms, 15 mm in length, were selected and fasted for at least for one week before sectioning. The operations on the worms were performed as follows. After the worms ceased to move as a result of being placed on a piece of wet filter paper on an ice plate, each was cut in a relaxed condition to generate sections of as uniform length as possible. The first transverse amputation was made through the mid portion of the prepharyngeal or postpharyngeal region to divide the worm into two parts. After various intervals the second amputation was made at a level 0.5 mm away from the first cut surface. Consequently, two groups of 0.5-mm-long fragments were prepared from the prepharyngeal and postpharyngeal regions; the first series consisted of fragments whose anterior cut surfaces after amputation were generated later than the posterior ones, referred to as *anterior later than posterior amputation*. The second series consisted of fragments whose posterior cut surfaces after amputation were generated later than the anterior ones, referred to as *posterior later than anterior amputation*. In *Dugesia japonica*, the organism

used in this study, it was confirmed by preliminary experiments that a length of 0.5 mm for the fragments was suitable. The survival ratio of the fragments declined markedly if they were made shorter than 0.5 mm in length, and longer fragments, for example, those of 1.0 mm or 1.5 mm in length, did not give rise to any bipolar heteromorphs.

The fragments, prepared as mentioned above, were kept in separate Petri dishes with aged tap water, maintained at $14 \pm 1^\circ\text{C}$. The water was changed every three days for one month. After the regeneration from the anterior and posterior cut surfaces was completed, the newly regenerated individuals were carefully examined under the binocular microscope and photographed.

RESULTS

Under the present condition ($14 \pm 1^\circ\text{C}$), a blastema occurred from the cut surface was observed about the fourth day and the eye in the blastema about the fifth day after amputation. The 0.5 mm long fragments, as showing in Tables, frequently produced bipolar heteromorphs. However, any bipolar heteromorphs were not obtained in the fragments of 1.0 mm and 1.5 mm in length made by the same way as those in the present experiment, of which particular data were not presented here.

Five types of the regenerate, namely, normal, no-head, no-tail, bipolar-head and bipolar-tail regenerates, were obtained. The normal type with a head at the anterior end and a tail at the posterior end was obtained predominantly in each experimental group. The no-tailed and no-headed types (Fig. 1a, b), in which regeneration of head or tail occurred only at one end of the body piece, were also found in each groups. However, these individuals were not considered to be a type of axial heteromorphs because they developed mainly as a result of simple fusion of the right and the left margins of the cut surface, preventing the formation of a blastema in one-sided amputation stump. The bipolar-head and bipolar-tail regenerates (Fig. 1c, d) are axial heteromorphs in the true sense, because the characteristics of one end of each was reversed with respect to the original body



FIG. 1. Four types of heteromorph that developed from isolated short fragments. a, No-head; b, no-tail; c, bipolar head; d, bipolar tail. $\times 30$.

polarity.

As summarized in Table 1 and 2, the 0.5 mm long fragments, which were isolated by two simultaneous transverse amputations from the pre- and postpharyngeal region of the worms, regenerated as normal worms in all cases examined. No-head and no-tail specimens occasionally occurred in every case of delayed second amputation, but it

appears that there is scarcely any relationship between the appearance of such worms and the interval between the two amputations. The appearance of bipolar heteromorphs, whether with bipolar heads or bipolar tails, was closely related to the interval between the first to the second amputation.

TABLE 1. Regeneration of fragments, of which posterior cut ends were made earlier than the anterior cut ends

Day of 2nd amputation	Number of fragments examined	Type of regenerate				
		Normal	No head	No tail	Bipolar head	Bipolar tail
<i>Isolated from the prepharyngeal region</i>						
0	18	18(100)	0(0)	0(0)	0(0)	0(0)
0.5	25	22(88)	0(0)	0(0)	3(12)	0(0)
1	32	21(66)	3(9)	5(16)	2(6)	1(3)
2	30	19(63)	5(17)	3(10)	0(0)	3(10)
3	25	25(100)	0(0)	0(0)	0(0)	0(0)
4	20	15(75)	2(10)	3(15)	0(0)	0(0)
6	58	55(95)	1(2)	2(3)	0(0)	0(0)
<i>Isolated from the postpharyngeal region</i>						
0	21	21(100)	0(0)	0(0)	0(0)	0(0)
0.5	48	40(84)	5(10)	1(2)	0(0)	2(4)
1	45	32(71)	7(16)	0(0)	0(0)	6(13)
2	43	33(77)	2(5)	4(9)	0(0)	4(9)
3	26	20(76)	1(4)	3(12)	0(0)	2(8)
4	25	22(88)	3(12)	0(0)	0(0)	0(0)
6	35	33(86)	2(14)	0(0)	0(0)	0(0)

The numbers in brackets are the percentages. Values indicated in boldface correspond to the cases of reversal of axial polarity.

TABLE 2. Regeneration of fragments, of which posterior cut ends were made earlier than the posterior cut ends

Day of 2nd amputation	Number of fragments examined	Type of regenerate				
		Normal	No head	No tail	Bipolar head	Bipolar tail
<i>Isolated from the prepharyngeal region</i>						
0	15	15(100)	0(0)	0(0)	0(0)	0(0)
0.5	48	41(86)	3(6)	2(4)	2(4)	0(0)
1	38	19(50)	0(0)	9(24)	10(26)	0(0)
2	46	26(57)	2(4)	9(20)	9(20)	0(0)
3	32	18(56)	1(3)	11(34)	2(6)	0(0)
4	23	19(83)	0(0)	4(17)	0(0)	0(0)
6	33	25(76)	0(0)	8(14)	0(0)	0(0)
<i>Isolated from the postpharyngeal region</i>						
0	18	18(100)	0(0)	0(0)	0(0)	0(0)
0.5	42	37(88)	1(2)	2(5)	2(5)	0(0)
1	46	40(87)	0(0)	1(2)	5(11)	0(0)
2	49	33(67)	0(0)	10(20)	6(12)	0(0)
3	23	19(83)	0(0)	3(13)	1(4)	0(0)
4	22	21(95)	0(0)	1(5)	0(0)	0(0)
6	46	43(94)	1(2)	2(4)	0(0)	0(0)

The numbers in brackets are the percentages. Values indicated in boldface correspond to the cases of reversal of axial polarity.

Anterior later than posterior amputation

The results are summarized in Table 1. In the fragments isolated from the prepharyngeal region, when the second amputation was delayed for 12 hours (0.5 day), bipolar-head regenerates were found in 3 (12%) out of 25 cases examined. With the second amputation delayed for one day, two (6%) bipolar-head and one (3%) bipolar-tail regenerate out of 32 cases were obtained. Furthermore, with the second amputation delayed for two days, bipolar-tail regenerate developed in three (10%) out of 30 cases. However, no bipolar heteromorphs were obtained from the fragments in which the second amputation was delayed for more than three days. In the fragments isolated from the postpharyngeal region, the bipolar-tail regenerates were obtained exclusively as heteromorphs as a result of the delayed second amputation. When the second amputation was delayed for 12 hours, two regenerates (4%) out of 48 were bipolar-tail. With the second amputation delayed for one day, 6 cases (13%) of bipolar-tail rege-

nerates out of 45 were obtained, which was the maximum percentage obtained. With amputations delayed still further, the incidence of these regenerates gradually decreased as the second amputation was delayed for three days. When the second amputation was delayed for more than four days no bipolar-tail regenerates were formed.

Posterior later than anterior amputation

The results are summarized in Table 2. When the posterior amputation was performed from 12 hours to three days after the first amputation, the bipolar-headed type of regenerate appeared. In the case of regeneration of fragments isolated from the prepharyngeal region, the rate of appearance of bipolar heads, brought about by the delay in the second amputation, was 2 (4%) out of 48 cases with a delay of 12 hours, and then the rate reached a maximum of 26% with a delay of one day, thereafter decreasing gradually to zero in the case of a delay of 4 days or more. In the case of regeneration of the fragments isolated from the postpharyngeal region, the pattern of occurrence

of the bipolar-headed type of regenerate was almost the same as that with the fragments isolated from the prepharyngeal region, that is, a delay in the second amputation of 12 hours gave 2 (5%) bipolar heads out of 42 cases. Subsequently, the incidence increased as the delay in the second amputations was prolonged, and the incidence reached a maximum of 12% with a delay of two days. Then the appearance of the bipolar heads decreased as the duration of the delay increased, and, when the second amputation was delayed for more than four days, no bipolar heads were obtained.

DISCUSSION

In regeneration of *Dugesia japonica*, within less than 24 hours of amputation needs the determination of the blastema to be a head or a tail under condition of about 20°C [10, 12], on which condition a blastema forms approximately at the second day after amputation. However, in the present experiments, the regeneration went on at a slow pace under low temperature. Therefore, the periods requiring for the determination of a blastema seem to be delayed. Such periods shall be seriously related to the production of bipolar heteromorphs from the short fragments made with the second amputation being delayed for some intervals after the first, as follows.

The 0.5 mm long body pieces that were long enough to be expected to maintain the original antero-posterior polarity when isolated as a result of two simultaneous amputations. It is noteworthy that the bipolar heteromorphs were developed when they were prepared with a limited interval of time between the two amputations. Such interval of time ranged from 12 hours to three days, when a blastema was not formed yet from the first cut surface. They appeared at the highest frequency with an interval of one or two days between amputations. Furthermore, the characteristics of the bipolar form, either bipolar-head or bipolar-tail, could be almost controlled at will by varying the order of the two amputations; the fragments for which anterior amputation was performed later than the posterior one tended to become bipolar-tail regenerates, while the fragments for which

posterior amputation was performed later than the anterior one tended to become bipolar-head regenerates. The formation of bipolar heteromorphs is clearly due to the interval between the two amputations. So, what occurred in the body pieces which were prepared with an interval? It is conceivable that an area organized previously for differentiation of either a head or a tail is established near the cut surface of the stump made by the first amputation prior to formation of a blastema there. That is, if the cut surface is situated at the anterior end of a section, a covert area with head-forming potential is established, and if the cut surface is situated at the posterior end of the sections, a covert area for a tail is established. The potential for regeneration of a head or tail in such covert areas increases with the passage of time. If the second amputation is made within the confines of the covert area, regeneration from the new cut surface will conform to preexisting developmental potential and, thus, some of the fragments isolated should show the mirror-image regeneration. In the case of short fragments made by two simultaneous amputations, covert areas with potential for head and tail coexist in the respective areas near the anterior and posterior cut surfaces at the same time. The effective range of the covert area seem not to extend far away. It is because almost of all the 1.0 mm and 1.5 mm long fragments normally regenerated, even though they were made in the same way as the 0.5 mm long fragments, as shown in our preliminary experiments.

Several fragments regenerated as the bipolar-headed type, in spite of belonging to the group in which posterior amputation was performed first. Such exceptional regenerates appeared in the case of pieces isolated from the prepharyngeal region, only if the second amputation was done within 24 hours, and not in the case of pieces from the postpharyngeal region. The occurrence of the bipolar head in this experimental group may be due to the fact that the capacity for producing a head is greater in the anterior region and decrease in the posterior direction along the body axis, as pointed out by Kanatani [10]. Accordingly, the covert area for forming a head initiated by the second amputation seems to have priority, because the second amputation was done before the tail-

forming covert area prepared by the first amputation was not fully established.

With an interval of more than four days between two amputations in the present experiments, any bipolar heteromorphs, either bipolar heads or bipolar tails, were not produced. The effect of such a delay in the second amputation clearly varied with the intervals between two amputations. It appears that, after a blastema to be a head or a tail is determined by the covert area, the potential as the covert area seems to disappear gradually, and the regenerating head or tail acts as a organizing center, one of which actions is to inhibit regeneration of identical characteristics in a feedback system, as shown in our previous experiments [6]. Child [19] lately found that the frequency of bipolar heads decreased with delays in amputation intervals, in which isolation of the fragments is almostly the same as in the present experiment, and he considered that the anterior cut end of the fragment, once determined as the position at which a head regenerates, dominates the posterior regeneration. However, as above mentioned, both cut ends coordinate the body patterning. Conceivably, the posterior cut end, once determined as the position as which a tail regenerates, is a second organizing area, in addition to the anterior cut end, both of which are determined by the covert areas, as suggested by the present experiment.

It is probably that, for the establishment of such covert areas with regenerative capacity, some dynamic changes in the cells included therein should occur near the cut surface. Indeed, remarkable increases in rates of synthesis of DNA, RNA and protein, and cell division are found in tissues near the cut surface [11, 20–22]. Although the determinative factors associated with the development of potential for formation of a head or a tail in the covert areas are known so far, it is clear that they are affected by the exposure of fragments to various inhibitors of protein synthesis and to anti-mitotic drugs [8, 10, 22–24], since treatment with such drugs causes the development of bipolar-head regenerates. Thus, at least one of the factors needed for establishment of the regenerating area is proteinaceous in nature and may be involved in cell division. By contrast, the nervous system is

known to play an important role in controlling the normal proportions of the body during regeneration; the fragments deprived of ventral nerve cords regenerated to be bipolar heads in high frequency [25–27] and the strips of nerve cord and cordless strips could not regenerate to be normal worms [28–30]. Although specific factors have not yet been found from the nervous system, it is quite possible that the patterning in planarian regeneration is closely related to the nervous system.

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